# Cysteine 29 is the major palmitoylation site on stomatin

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**Abstract The 31 kDa membrane protein stomatin was metabolically labeled with tritiated palmitic acid in the human amniotic cell line UAC and immunoprecipitated. We show that the incorporated palmitate is sensitive to hydroxylamine, indicating the binding to cysteine residues. Stomatin contains three cysteines. By expressing a myc-tagged stomatin and substituting the three cysteines by serine, individually or in combination, we demonstrate that Cys-29 is the predominant site of palmitoylation and that Cys-86 accounts for the remaining palmitate labeling. Disruption of Cys-52 alone does not show any detectable reduction of palmitic acid incorporation. Given the organization of stomatin into homo-oligomers, the presence of multiple palmitate chains is likely to increase greatly the affinity of these oligomers for the membrane and perhaps particular lipid domains within it.**



*Key words:* Membrane protein; Amniotic cell; Microvillus

## 1. Introduction

The integral membrane protein stomatin was first recognized as an important component of the erythrocyte membrane (band 7.2b). After its subsequent purification [1,2], and the cloning of its cDNA [3,4], the primary structure revealed a 29 amino acid hydrophobic stretch, the putative membrane domain, preceded by a 24 residue highly charged N-terminus and followed by the C-terminal part containing the majority of the 287 residues. Both N- and C-terminal portions of the molecule are thought to be exposed at the cytoplasmic face of the lipid bilayer [2,5]. Stomatin expression is not restricted to red cells and can be detected in many different tissues and cell lines [2,4,6,7].

Since our finding of the 4–6-fold up-regulation of stomatin in the human amniotic cell line UAC after treatment with interleukin-6 (IL-6) and dexamethasone [8], we have been using this model to characterize the cellular localization as well as some structural aspects of this protein. We have shown that stomatin is concentrated in plasma membrane protrusions and actin rich structures in UAC cells [9]. It is also present in abundance in a population of juxta-nuclear vesicles. We then demonstrated that the macromolecular organization of stomatin is homo-oligomeric. Large oligomeric complexes of stomatin can be extracted from cellular membranes using the non-ionic detergent Triton X-100; nevertheless, a significant proportion of the protein remains connected to the cytoskeleton [10]. The complexes have an approximate size of 300 kDa (9–12 monomers). As two C-terminally truncated forms of stomatin are not incorporated into the complexes, it seems likely that the C-terminus of the protein is involved in their formation.

The presence of high order oligomers of stomatin on the cytoplasmic side of plasma membrane folds, its partial association with the cytoskeleton as well as its co-localization with cortical actin microfilaments strongly point to a structural role and suggest that stomatin can have an influence on the cortical morphogenesis in UAC cells and perhaps other cells.

An important feature of stomatin is the presence of three cysteine residues in positions 29, 52 and 86. Two of the residues (Cys-29 and Cys-52) reside at the start and end of the membrane domain whilst the third cysteine (Cys-86) is located in a region characterized by a stretch of non-polar amino acids. The proximity of these residues to the plasma membrane makes them possible candidates for palmitoylation by thioester linkage. This important post-translational lipid modification occurs alone or in combination with other acylation types on different integral and peripheral membrane proteins. Examples are found, among others, in membrane receptors, α subunits of heterotrimeric G proteins, small GTPases, and non-receptor tyrosine kinases [11–13]. Palmitoylation confers greater membrane affinity, but can also affect a protein functionally or influence its interaction with other proteins and specific membrane domains.

In this report, we show that stomatin is palmitoylated in UAC cells and that this acylation occurs by thioester linkage on cysteine residues. Analysis of the palmitoylation sites by directed mutagenesis of the three cysteines indicates that Cys-29, located near the N-terminal end of the membrane domain, is the major site of palmitoylation on stomatin whereas Cys-86 contains all or most of the remaining palmitic acid.

## 2. Materials and methods

### 2.1. Reagents

The monoclonal antibody GARP 50, used directly from hybridoma supernatant, specifically recognizes stomatin and has been described previously [2,9,10]. Hydroxylamine and anti-mouse agarose were from Sigma. Palmitic acid [9,10-$^3$H(N)] was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA).

### 2.2. Cell culture

UAC cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Belgium) supplemented with 10% fetal calf serum (Life Technologies, Inc.). To induce maximal expression of stomatin, cells were grown to confluence and treated for 24 h with 200 U/ml IL-6 and 0.1 μM dexamethasone as described [8].

### 2.3. DNA mutagenesis and cell transfections

To express recombinant stomatin in UAC cells, we used the eukaryotic expression vector pEF-Puro.PL3 modified to allow fusion of stomatin with the myc-tag epitope at the C-terminus, as described previously [10], with the exception that only one copy of the myc epitope was added in this study. From this construct, an *Eco*RI-*Not*I fragment containing the stomatin-myc fusion was inserted into the vector

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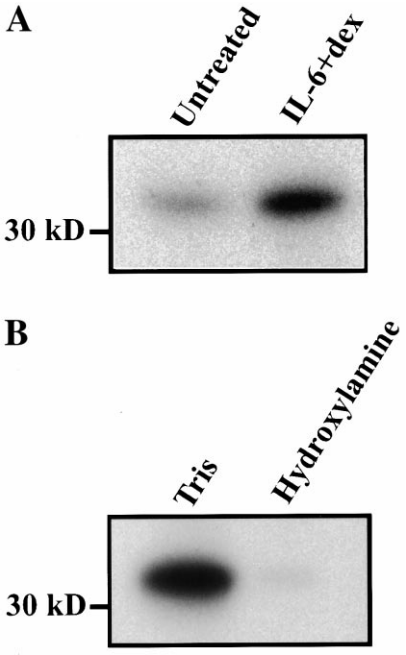


Fig. 1. Palmitoylation of stomatin on one or more cysteine residues. A: UAC cells were treated with IL-6 and dexamethasone or left untreated as indicated. They were metabolically labeled with [$^3$H]palmitic acid before immunoprecipitation of stomatin. B: [$^3$H]Palmitate labeled stomatin was immunoprecipitated from UAC cells. Half of the precipitate was treated with 1 M Tris-HCl pH 7.2 (Tris) and the other half was treated with 1 M hydroxylamine pH 7.2 (Hydroxylamine).

pcDNA$_3$ (Invitrogen). This latter plasmid was used to generate Cys to Ser (TGC to AGC) mutations at positions 29, 52 and 86 of the stomatin amino acid sequence. This was accomplished by overlap extension using complementary oligonucleotides carrying the desired mutations and T7 and SP6 primers flanking the multiple cloning site of pcDNA$_3$. All mutations were checked by dideoxy sequencing. Vectors containing the single mutants were used as templates to generate double mutants and one of those served to produce the triple mutant. Constructs in pcDNA$_3$ were transfected transiently in UAC cells (ProFection Mammalian Expression System, Promega) to verify expression of the respective proteins by immunofluorescence using the anti-myc antibody (Boehringer Mannheim). Subsequently, for each individual construct, a *Spe*I-*Eco*RV fragment comprising the mutagenized stomatin was subcloned into pEF-Puro.PL3. The resulting vectors were used to generate stable transfectants in UAC cells as described [10].

*2.4. Palmitate labeling and immunoprecipitations*

For metabolic labeling with radioactive palmitic acid, cells were grown to confluence in 10 cm petri dishes. They were rinsed twice with serum-free medium and incubated for 4 h in 5 ml of serum-free medium containing 300 μCi of tritiated palmitate. After incubation, cells were rinsed two times with PBS, lysed in 1 ml of TNET (20 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin and 5% fetal calf serum. After removal of insoluble material by centrifugation, lysates were incubated successively with anti-stomatin antibody and goat anti-mouse IgG agarose. Immune complexes were recovered by centrifugation and washed four times with TNET. They were then eluted in Laemmli sample buffer (without β-mercaptoethanol) and separated by SDS-PAGE. Gels were treated with a fluorographic agent according to the manufacturer's instructions (Amplify, Amersham), dried and exposed on Kodak BioMax MR films. Exposure times ranged from 10–20 days (Fig. 1) to 2 months (Figs. 2 and 3). Scanning densitometry of autoradiographs was performed on a Personal Densitometer (Molecular Dynamics) using the program ImageQuant.

*2.5. Hydroxylamine treatment*

IL-6 and dexamethasone treated UAC cells were metabolically labeled with tritiated palmitate and stomatin was immunoprecipitated. Half of the precipitate was rotated overnight in a solution of 1 M hydroxylamine, pH 7.2, while the other half was incubated in 1 M Tris-HCl, pH 7.2. Subsequently, the solutions were discarded. Proteins were eluted in Laemmli sample buffer and separated by SDS-PAGE. Labeling of stomatin was visualized by fluorography.

*2.6. Immunoblotting*

Extracts of the different clones, obtained concomitantly by the same procedure as for immunoprecipitation, were analyzed by Western blotting as described [10], using anti-stomatin antibody GARP 50, anti-mouse horseradish peroxidase conjugate (Promega) and detection by chemiluminescence (SuperSignal Chemiluminescent Substrate, Pierce).

## 3. Results and discussion

*3.1. Stomatin is palmitoylated by thioester linkage*

To investigate the possibility that stomatin is palmitoylated in UAC cells, we labeled the cells metabolically with tritiated palmitic acid and immunoprecipitated stomatin after lysis in a buffer containing 1% Triton X-100. This detergent has been shown to solubilize 70–80% of stomatin from these cells [10]. Immunoprecipitates were analysed by SDS-PAGE and fluorography. Fig. 1A shows that immunoprecipitated stomatin has incorporated palmitate. In IL-6 and dexamethasone treated cells, there is an increase of labeling corresponding to the reported up-regulation of stomatin following this treatment [8]. This last experiment indicates that the degree of palmitoylation of the protein remains constant even when its expression is increased.

Next, we examined the sensitivity of stomatin-bound palmi-

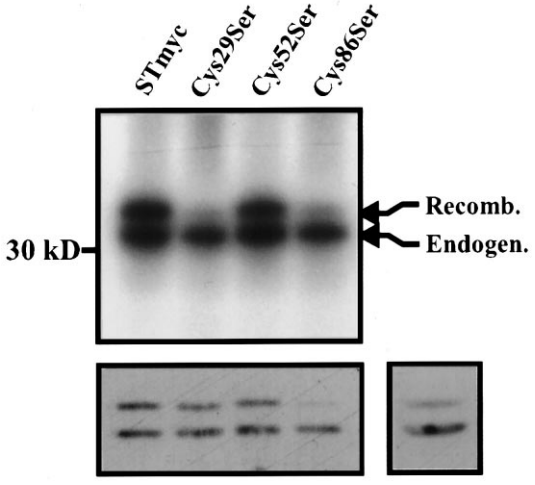


Fig. 2. Cys-29 is the major palmitoylation site on stomatin. Upper panel: UAC cells stably expressing stomatin C-terminally fused to a myc tag (STmyc), or the mutated forms Cys-29-Ser, Cys-52-Ser and Cys-86-Ser of this construct, as indicated, were labeled with tritiated palmitic acid and subjected to immunoprecipitation using the anti-stomatin antibody. Note that cells were not treated with IL-6 and dexamethasone to avoid a too strong signal from endogenous stomatin. The gel was exposed for 2 months in order to visualize weakly labeled bands. Arrows indicate the positions of endogenous stomatin (Endogen.) or myc-tagged stomatin and mutants (Recomb.). Lower panel: At the same time, extracts of the different clones were analyzed by immunoblotting using the anti-stomatin antibody. To see better the expression of Cys-86-Ser, an immunoblot was performed with more protein (lower panel, inset).

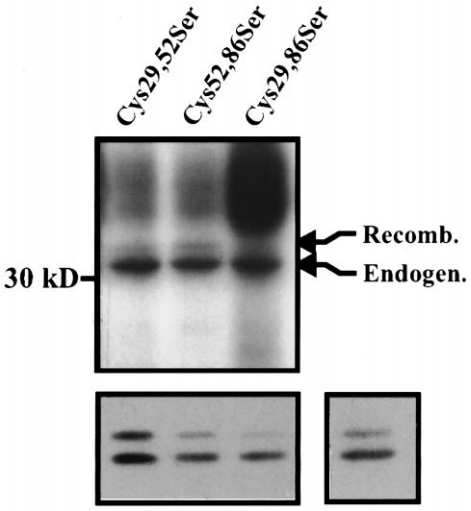


Fig. 3. Stomatin Cys-29,86-Ser is not palmitoylated. Upper panel: UAC cells expressing the double mutants Cys-29,52-Ser, Cys-52,86-Ser and Cys-29,86-Ser were metabolically labeled with tritiated palmitic acid and immunoprecipitations were performed using anti-stomatin antibody. Arrows indicate the positions of endogenous stomatin (Endogen.) or myc-tagged stomatin and mutants (Recomb.). Lower panel: Extracts from the same cells were analyzed by immunoblotting using anti-stomatin antibody. The inset shows an immunoblot of the Cys-29,86-Ser extract, with more protein loaded on the gel to show better the presence of the recombinant protein.

tate to neutral hydroxylamine treatment. Fig. 1B shows that the incorporated palmitic acid is almost completely released by incubating the immunoprecipitate in 1 M hydroxylamine pH 7.2, as compared to 1 M Tris at the same pH. This result demonstrates that stomatin undergoes acylation through thioester linkage involving one or more cysteine residues.

### 3.2. *Cys-29 is the major palmitoylation site*

In order to determine which of the three cysteines at positions 29, 52 and 86 is palmitoylated, we substituted each cysteine with serine, either individually, two at a time or all three together, and expressed these mutated stomatin molecules in UAC cells. The expressed proteins were fused at the C-terminus to a myc epitope allowing us to distinguish between recombinant and endogenous stomatin by SDS-PAGE and also making possible the examination of cellular localization by immunofluorescence. We have shown previously that the myc-tagged stomatin displays immunofluorescence patterns identical to the endogenous protein and can efficiently incorporate into stomatin oligomers [10]. In transient transfections followed by immunofluorescent staining using the anti-myc antibody, all the different mutants were found to be localized as stomatin (not shown). However, the triple Cys-29,52,86-Ser mutant was expressed in much fewer cells and was more difficult to detect, probably because of a greater instability of the molecule. For palmitic acid labeling experiments, we performed stable transfections of the different constructs, because stable transfections allow recombinant proteins to be more evenly expressed among cells of the same culture. First, clones expressing each mutated stomatin were isolated and analysed by Western blotting using the anti-stomatin antibody (Figs. 2 and 3). For Cys-86-Ser, Cys-52,86-Ser and Cys-29,86-Ser, we could only obtain low expression levels compared to the other constructs. We did not obtain clones stably expressing the Cys-29,52,86-Ser mutant. We then metabolically labeled cells expressing wild-type, Cys-29-Ser, Cys-52-Ser and Cys-86-Ser epitope-tagged stomatin with palmitate and lysates were immunoprecipitated using the anti-stomatin antibody. For each clone, the endogenous stomatin was used as a control of labeling efficiency (Fig. 2). The data show that, while the palmitoylation of myc-tagged stomatin is as efficient as the endogenous protein, labeling of Cys-29-Ser is strongly reduced (as compared to the expression level assessed with the immunoblot). Cys-52-Ser does not display any apparent reduction of palmitoylation and Cys-86-Ser palmitoylation is only slightly reduced, relative to the expression level. Quantitative analysis of the autoradiographs by scanning densitometry (Table 1) shows that the radioactivity associated with Cys-29 comprises 70–85% of the total labeling. It also indicates that the remaining palmitoylation (15–30%) occurs on Cys-86. To confirm these data, we performed the same experiment using the double mutants (Fig. 3 and Table 1). The strong decrease of labeling observed for Cys-29,52-Ser is in agreement with the result obtained for Cys-29-Ser. For Cys-52,86-Ser, the protein is still clearly radiolabeled despite its low expression. Finally, no radioactive band can be observed for Cys-29,86-Ser even after a very long exposure of the gel. Because of the background in Fig. 3, upper panel, third lane, we performed an additional palmitate labeling of Cys-29,86-Ser. The result confirmed that this mutant is not palmitoylated (not shown). In conclusion, experiments presented in Figs. 2 and 3 and summarized in Table 1 demonstrate that Cys-29 is the major site of palmitoylation of stomatin (70–85%) and that the remaining acylation occurs on Cys-86.

Palmitoylation of stomatin is not restricted to UAC cells, as it was also reported for band 7.2b in erythrocytes [1]. A prerequisite for this modification is the proximity of a cysteine residue to the membrane. This is the case for Cys-29 in stomatin and probably also for Cys-86, which is preceded by a short hydrophobic stretch. The possibility has been raised that this region constitutes a second membrane spanning domain in UNC-1, a protein very similar to stomatin in *Caenorhabditis elegans* [14]. Our finding that Cys-52 might be completely devoid of palmitoylation does not contradict this model, but digestion studies performed on intact erythrocytes exclude in principle an extracellular part for stomatin [2]. At any rate, even a partial palmitoylation of Cys-86 assigns this region to the membrane vicinity while palmitoylation of Cys-29 confirms, if needed, that the N-terminus of stomatin is intra-cytoplasmic [5].

Table 1
Quantitative analysis of stomatin palmitoylation

| | WB | AR | AR/WB |
|---|---|---|---|
| STmyc | 95 | 101 | 106 |
| Cys-29-Ser | 74 | 11 | 15 |
| Cys-52-Ser | 74 | 82 | 111 |
| Cys-86-Ser | 22 | 15 | 68 |
| Cys-29,52-Ser | 59 | 9 | 15 |
| Cys-52,86-Ser | 30 | 20 | 67 |
| Cys-29,86-Ser | 17 | 0 | 0 |

Autoradiographs and Western blots shown in Figs. 2 and 3 were analyzed by scanning densitometry. WB, AR: percentages of expression (WB) or of palmitoylation (AR) of the myc-tagged stomatin (or the different mutants) as compared to endogenous stomatin. AR/WB: values obtained for the autoradiographs are given as a percentage (±10%) of the values obtained for the Western blots.

We have already compared stomatin to caveolin [10]. Both proteins are anchored to the cytosolic part of the membrane by a ''hairpin loop'' and also build up high order oligomers [10,15,16]. The palmitoylation of stomatin resembles caveolin in that it occurs on more than one cysteine and the major acylation site (Cys-133 in caveolin) is located at one end of the membrane domain, on the side of the shorter cytoplasmic stretch [17]. Interestingly, both Cys-29 in stomatin and Cys-133 in caveolin are flanked by a proline residue. This is also the case for Cys-86 of stomatin. That an adjacent proline and a hydrophobic environment might be favorable to trigger palmitate addition to a cysteine is a possibility which was suggested for the Rh protein [18].

The organization of stomatin into oligomers does not allow us to examine whether abolition of palmitoylation has an impact on the protein affinity for the membrane, because oligomers also contain the endogenous palmitoylated protein. This can explain why all the mutants were found to have a correct cellular localization. Nevertheless, some cysteine residues appear to be important for stomatin stability, without correlation with the palmitoylation level. This seems to be the case for Cys-86 because proteins mutated at this residue could be expressed stably only in low amounts, and also for Cys-52, as the triple mutant could not be stably expressed, in contrast to Cys-29,86-Ser. These low or undetectable expression levels are likely to be a consequence of instability of the proteins at early stages of biosynthesis.

Although experiments described in this paper do not allow us to deduce the stoichiometry of stomatin palmitoylation, one can assume that the organization of this protein into stable oligomers covalently linked to several palmitate chains creates a relatively high concentration of this lipid in the plasma membrane. In an interesting discussion on caveolin, Brown and London [19] propose that this effect could be one reason why this protein has privileged interactions with detergent insoluble lipid domains [20]. Our preliminary data indicate that stomatin is partially present in detergent resistant domains in UAC cells along with GPI-anchored proteins with which it co-localizes in immunofluorescence (L. Snyers, unpublished results). This property is likely to represent one rationale for the covalent attachment of palmitic acid to stomatin.

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